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(54) Title: NOVEL DRUG DELIVERY SYSTEMS FOR PROTEINS AND PEPTIDES USING ALBUMIN AS A CARRIER MOLECULE

(57) Abstract

A drug delivery system for proteins and peptides using albumin as a carrier molecule. The drug is bound noncovalently to albumin by means of an attached apolar substituent, preferably a long chain fatty acid derivative.

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NOVEL DRUG DELIVERY SYSTEMS FOR PROTEINS AND PEPTIDES USING ALBUMIN AS A CARRIER MOLECULE

BACKGROUND OF THE INVENTION

This invention relates to a novel drug carrier system to enhance the efficacy of peptides and proteins used as therapeutic agents. Improvements in the chemical methods for peptide synthesis and the use of recombinant DNA techniques to produce larger polypeptides and proteins have greatly expanded the therapeutic uses of these drugs. Representative applications include the use of superoxide dismutase (SOD) as an anti-inflammatory and anti-ischemic agent, the soluble CD4 protein for the treatment of AIDS, polypeptide hormones such as human insulin, gamma-interferon and other lymphokines for the treatment of infectious diseases and certain types of cancer, growth factors such as growth hormone and erythropoeitin which is useful in the treatment of anemia, and tissue plasminogen activator useful as a thrombolytic agent. A further advantage of the new methods of synthesis of these peptides and proteins is that problems of immunogenicity, which often arise if the molecule is isolated from an animal source, can be avoided by producing the native human protein.

However, one common limitation in the use of proteins and peptides as drugs is that they are rapidly metabolized or excreted in the urine. The renal threshold for filtration of molecules dissolved in plasma is in the range of 50,000 to 60,000 daltons. Smaller molecules are very rapidly filtered from the circulation by the kidneys. For example, SOD with a molecular weight of only 32,000 daltons has a half-life in plasma of only about 6 minutes. Hemoglobin normally a tetramer of two

weight of just 32,000. At typical therapeutic concentrations when acellular hemoglobin is used as a blood replacement, about 3 to 5 percent of the protein exists as dimers. As in the case of SOD, the dimers are cleared from the circulation with a half-life on the order of minutes. As the concentration of dimers becomes depleted the equilibrium

$$\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta \quad (1)$$

becomes progressively shifted to the right. The newly formed dimers are likewise excreted. This process continues until all of the hemoglobin is eliminated. The overall half-life of hemoglobin in the circulation is about 90 minutes. Not only does this compromise its function as a blood substitute, but the massive dumping of protein in the urine also poses the risk of renal injury.

Several approaches have been taken to prolong the intra-vascular retention time of such molecules, all of which involve extensive covalent modification of the protein. A variety of nonspecific cross-linking agents have been employed to polymerized hemoglobin to produce higher molecular weight species (see for example Bonsen, U.S. Patent 4,001,401). Most commonly, glutaraldehyde has been used which reacts more or less randomly with the amino groups of the 44 lysine residues and 4 amino-termini of the hemoglobin molecule. This results in a polydisperse mixture which appears at best to be of limited clinical utility. Alternatively, hemoglobin and other proteins of therapeutic interest have been covalently cross-linked to a macromolecular carrier such as dextran or albumin.

Artificial Cells, and Artificial Organs 16, 237-245; Poznansky, M.J. (1988) Methods in Enzymology 137, Academic Press, New York, pp. 566-574). A third approach which has been taken to increase the effective size of polypeptide and protein drugs, and thereby prevent their rapid renal excretion, is to decorate the molecule with multiple copies of a lower molecular weight substance. Polyethylene glycol in the molecular weight range of 2,000 to 10,000 daltons has been used most frequently for this purpose, (Abuchowski, A. et al., (1977) Journal of Biological Chemistry 252, 3578-3581, 3582-3586; Pyatak, P.S. et al. (1980) Research Communications in Chemical Pathology and Pharmacology 29, 113-127).

The extensive modification of the protein that results using all three of these methods invariably leads to a very heterogenous mixture of products. The composition can be characterized only by certain average properties making batch to batch uniformity difficult to insure. Extensive modification of the protein or peptide can also lead to a decrease or loss of activity; and especially in the case of cross-linking agents, problems of antigenicity can arise. To date, there are no protein or peptide cross-linked conjugates that are in use clinically; and there are only a few examples in which decoration with polyethylene glycol has lead to the development of a useful therapeutic agent, despite the introduction of this approach nearly 15 years ago. Thus, while some progress has been made, there remains a continuing need for improved delivery systems for protein and peptide drugs. The primary

A yet further objective of the present invention is to provide a drug carrier system for proteins and peptides in which such molecules are modified at a unique or a limited number of sites with an apolar (hydrophobic) substituent to serve as a bridging group to mediate binding of the drug noncovalently to albumin.

A yet further objective of the invention is to provide reagents which can be used to selectively modify peptides and proteins with fatty acid derivatives.

A yet still further objective of the present invention is to provide a means of regulating the activity of peptides and proteins used therapeutically by competition with free fatty acids for binding sites on albumin, resulting in displacement of the drug.

SUMMARY OF THE INVENTION

A new drug delivery system for peptides and proteins wherein such molecules are modified with apolar substituents to promote noncovalent binding to albumin, which in turn serves as a macromolecular carrier for the drug within the circulation. In the most preferred embodiment of the invention the protein is modified at a single or limited number of sites with a long chain fatty acid derivative. Novel protein modifying agents to attach such groups are provided. A method for regulating the activity of such drugs based on competition for binding sites on albumin with free fatty acids is also presented.

DETAILED DESCRIPTION OF THE INVENTION

the present invention albumin is used as a carrier molecule for the transport of peptide and protein drugs within the circulation. The invention is especially useful for polypeptides below a molecular weight of about 60,000 which normally would be rapidly excreted in the urine. Biding of the protein to albumin is mediated by an attached derivative. Long chain fatty acid derivatives are the preferred bridging groups to provide high binding affinity.

The molecular weight of the albumin is 66,000 daltons. Normally very little of the protein (less than 100 mg/day) is excreted in the urine. Albumin plays an important role physiologically in the transport of free fatty acids within the circulation. There are at least 10 sites on the protein at which fatty acids can bind. The two highest affinity binding sites are selective for fatty acids. The remaining binding sites can accommodate a variety of apolar molecules, particularly organic anions. The association constant for the binding of long chain fatty acids such as palmitate (C_{16}) and stearate (C_{18}) at the high affinity sites is on the order of 10^8 M^{-1}. There are 3 to 5 secondary sites for which the binding constant is about 10^6 M^{-1}, still relatively large. The binding energy is due primarily to hydrophobic interactions. A smaller, but significant contribution is due to electrostatic interactions between the carboxylate group and a surface lysine residue of the protein adjacent to the apolar binding pocket. The carboxylate group can be replaced with other negatively charged substituents, including but not

affinity. The molar ratio of free fatty acids to albumin in the blood is typically about 0.6. At the highest plasma concentration of free fatty acids observed physiologically, which occurs during strenuous exercise, the molar ratio is 4. Thus under all conditions there are vacant binding sites on the protein with affinities for free fatty acids of at least 10^6 M^{-1}. For further discussion of the structure and function of albumin see Spector, A.A. (1986) in Biochemistry and Biology of Plasma Lipoproteins (Scanu, A.M. and Spector, A.A., eds.) Marcel Dekker, Inc., New York, pp. 247-279, which is incorporated herein by reference.

A variety of apolar molecules can be used as the bridging group to promote the binding of peptides and proteins to albumin. Organic anions are preferred. The most preferred bridging groups are long chain fatty acid derivatives. The length of the hydrocarbon chain can vary from about 10 to about 24 carbon atoms, preferably 16 to 20 carbon atoms. Carbon-carbon double bonds, such as in derivatives of oleic acid, carbon-carbon triple bonds, and heteroatoms uncharged at physiological pH, including but not limited to oxygen and sulfur, can also be incorporated into the chain. A number of different negatively charged groups may be introduced into the molecule to enhance the binding affinity to albumin, including carboxylates, phosphates, phosphonates, sulfates and sulfonates. Functional groups which may be incorporated into the reagent to provide a means of attachment to the protein include: (1) activated carboxylic acid derivatives such as anhydrides, N-hydroxy-

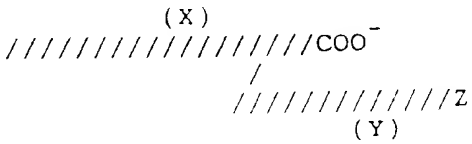
acids and amides or N-substituted maleimides; and (3) carbonyl groups, aldehydes and ketones, which may be used to link the compound to amino groups of the protein by reductive alkylation. For further discussion of the reactive groups which may be used to covalently modify different amino acid residues of proteins and peptides see Lundblad, R.L., and Noyes, C.M. (1984) Chemical Reagents for Protein Modification, CRC Press, Boca Raton, Florida, which is incorporated herein by reference.

In the preferred practice of the invention the attachment of the apolar group is restricted to a unique site or a limited number of positions on the protein or peptide. This makes it possible to produce a specific derivative having well defined and desirable properties, in contrast to the random mixture of reaction products which results from cross-linking proteins and peptides to macromolecular carriers. The reagent may be directed to a specific region of the molecule on the basis of noncovalent binding interactions at the site.

Example 2 below illustrates the use of such an affinity reagent to modify hemoglobin with a long chain fatty acid derivative selectively at the 2,3-diphosphoglycerate binding pocket located at the interface between the beta chains. Alternatively, the sites of modification may be restricted by using reagents that react at the SH group of cysteine residues. Cysteine is uniquely reactive among the amino acid residues naturally found in proteins and can be readily targeted with a number of different alkylating agents such as alpha-halo carboxylic acids and amides and maleimide derivatives (see

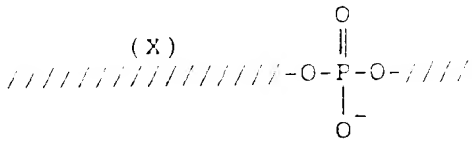
residues. In hemoglobin there are three: beta 93, beta 112 and alpha 104 (six in total per tetramer). Beta 112 and alpha 104 are buried inside the molecule; only cysteine beta 93 is reactive. In SOD there are four cysteines per 32,000 molecular weight dimer; only the two cysteine 111 residues are reactive. In proteins in which a cysteine residue is absent, one may be readily introduced using recombinant DNA techniques. For shorter polypeptides, synthetic methods can be used to replace one of the native amino acids within the sequence with cysteine, or an additional cysteine residue can be added to the molecule. There are, of course, certain amino acid residues that are critical for the activity of any given protein which cannot be modified, but generally there are a number of positions at which amino acid substitutions can be made with relatively little effect on the properties of the molecule.

Two novel classes of modifying agents have been developed which can be used to attach fatty acid derivatives to proteins and peptides:



(I)

and



(II)

In (I) the negatively charged substituent is provided by a carboxylate group, in (II) by a phosphate group. The long chain apolar group (X) which will

atoms, preferably 16 to 20 atoms. Heteroatoms such as oxygen and sulfur, as well as other apolar functionalities may be incorporated into the chain. The spacer arm (Y) through which the fatty acid derivative is attached to the protein or peptide can be a simple aliphatic chain, or it may incorporate polar or charged groups to enhance water solubility. Aromatic substituents may also be incorporated into the spacer arm. The functional group (Z) provides the means of attachment of the reagent to the protein or peptide. It may be any of the reactive groups used to modify proteins described above. Syntheses of representative examples of these two classes of compounds is described in Example 1. Example 2 demonstrates the reaction of these reagents with hemoglobin.

Given the high concentration of albumin within plasma (about 0.65 mM) and the multiple number of available binding sites, an association constant of only 10^5 M^{-1} will ensure that over 99% of a fatty acid derivatized protein or peptide will be bound to albumin. Even the secondary binding sites have association constants of 10^6 M^{-1} for free fatty acids. Because the concentration of albumin will generally be in vast excess over the therapeutic level of the protein or peptide drug, a 1:1 complex between albumin and the drug molecule will be formed; i.e. there will be a negligible fraction of species in which two or more molecules of the drug are bound to the same molecule of albumin. One important exception to this is in the use of hemoglobin as a blood substitute. In this case, the plasma concentration of hemoglobin is on the order

higher molecular weight species in which there are two or more molecules of hemoglobin attached to a single molecule of albumin can be formed.

Not only is renal excretion of the protein bound to albumin blocked, but filtration across other capillary beds is also limited. This is particularly true of hemoglobin blood substitutes bound to albumin due to the higher molecular weight species that are formed. Binding of the protein to albumin can also enhance lymphatic return from the interstitial fluid. Even after cross-linking hemoglobin intramolecularly to prevent dissociation of the tetramer the intravascular retention time is much shorter than that of albumin, despite the fact that both proteins have nearly the same molecular weight. The initial rate of egress of the two proteins from the circulation is, however, nearly the same. Whereas nearly all of the albumin filtered is returned to the blood via the lymphatics, very little of the hemoglobin is recovered.

Protein and peptide drugs used in accordance with the present invention are generally administered parenterally. Co-administration of the drug with albumin may be employed but is usually not required. Binding of the modified protein or peptide occurs rapidly to the endogenous albumin within the circulation, or to albumin within the interstitial fluid if the drug is given subcutaneously or intramuscularly. Replacement of albumin may become necessary in the treatment of severe hemorrhage when using fatty acid modified hemoglobin derivatives as blood substitutes. Suitable pharmaceutical carriers for the modified protein

saline, a mixture of glucose and saline or Ringer's lactate. Conventional methods for administering such therapeutic agents are known medical state of the art, see for example, Avis, K. (1985) in Remington's Pharmaceutical Sciences (Gennaro, A.R., ed.) Mack Publishing Company, Easton, PA, pp. 1518-1541, which is incorporated herein by reference.

Proteins or peptides modified with apolar substituents may remain fully active while associated with albumin. Such is the case for SOD and hemoglobin derivatives, for example. Alternatively, only the free polypeptide may retain activity, in which case the complex with albumin would provide an inactive depot form of the drug. Since the vast majority of the protein is bound to albumin, this provides an effective reservoir to buffer the free concentration of the drug. As the free form of the drug becomes metabolized or excreted, the equilibrium in equation (2) shifts progressively to the right, i.e. the protein becomes released from albumin. This maintains the concentration of the free drug, and hence the level of activity, in a relatively narrow range between doses.

$$\text{Albumin Protein} \rightleftharpoons \text{Albumin} + \text{Protein} \qquad (2)$$

Using albumin as a carrier molecule for protein and peptide drugs offers further advantages. Association of the drug with albumin may protect it from proteolytic or other enzymatic degradation. Since the binding of the protein to albumin is reversible free fatty acids or other apolar molecules can be administered to displace the protein from the complex by competition for the

very rapidly excreted in the urine, i.e. on the order of minutes, this will abruptly terminate the activity of the agent. This provides a novel means by which the duration of action of protein and peptide drugs can be precisely controlled.

The present invention also affords a novel class of long acting insulin derivatives which bind to albumin by means of an attached apolar group. Competition with endogenous free fatty acids gives rise to particularly useful pharmacokinetic properties for these derivatives. Normally there is an excess of available binding sites on albumin so that the modified insulin is almost 100% in the bound form. In this state, the insulin is inactive. Regular insulin is given intermittently to maintain normal metabolic control. If the levels of insulin administered are inadequate, the concentration of free fatty acids in the blood increases. This displaces some of the insulin bound to albumin and ameliorates the insulin deficiency. Such apolar modified insulin derivatives are particularly useful in preventing the complications of diabetic keto-acidosis, in which case the free fatty acid concentration can be as high as 3 to 4 mM.

The following examples are offered to further illustrate, but not limit, the process, products and medical techniques of the invention.

Example 1

Synthesis of Novel Fatty Acid Protein and Peptide Modifying Agents

Sodium 1-hexadecyl, 6-(2-iodoacetomido)hexyl phosphate (5)

This new compound was synthesized by the following 5 step sequence. In the first step 1-hexadecanol (2.42 g, Aldrich) was condensed with 2-cyanoethyl-N,N-diisopropylamino chlorophosphine (2.84 g, American Bionetics) in dry methylene chloride in the presence of 2.58 g of diisopropylethylamine. The reaction was complete within 10 minutes. The mixture was poured into ethyl acetate and was washed first with saturated sodium bicarbonate, then with brine. The solution was then dried with sodium sulfate and the solvent was evaporated. The crude material was chromatographed over silica gel using 25% ethyl acetate:65% hexanes:10% triethylamine as the solvent to isolate the product: 1-hexadecanyl, 2-cyanoethyl N,N-diisopropylphosphoramidite (1), as a colorless liquid.

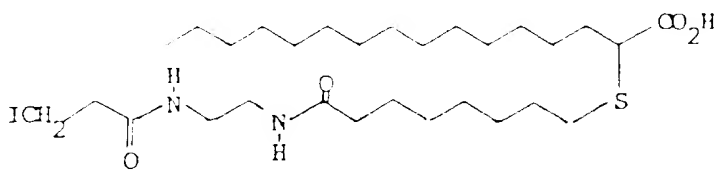
Compound 1 (220 mg) was next coupled to N-(p-anisyldiphenylmethyl)-6-aminohexanol (194mg, prepared as described by Connolly, B.A., Nucleic Acids Research (1987) 15, 3131-3139). The reaction was carried out in dry acetonitrile in the presence of tetrazole (2 equivalents) as an activator. After 15 minutes, the reaction mixture was evaporated to dryness, resuspended in hexanes and then chromatographed over silica gel. The product: 1-hexadecyl,2-cyanoethyl N-(p-anisyldiphenylmethyl)-6-aminohexyl phosphite (2) was eluted with 84% hexanes:15% ethyl acetate:1% triethylamine as a colorless oil.

Next 2 was oxidized with t-butyl hydroperoxide (2.2 equivalents) to afford the corresponding phosphate (3). The reaction was carried out in methylene chloride for 10 minutes. The solvent was

The 2-cyanoethyl group was next removed by refluxing 3 in triethylamine:acetonitrile (1:1) for 5 hours. After removal of the solvents, the p-anisyldiphenylmethyl group was removed by treatment with 80% aqueous acetic acid at room temperature for 3 hours. The acetic acid was then removed by evaporation. The residue was dissolved in water and washed with hexanes to remove p-anisyldiphenylmethyl alcohol. The aqueous layer was then evaporated and the product 1-hexadecyl, 6-aminohexyl phosphate (4) was isolated as a white precipitate by the addition of ethyl ether.

Finally, the title compound 5 was obtained by the reaction of 4 with iodoacetic anhydride. Compound 4 (210 mg) was dissolved in 3.0 ml of 1.0 N NaOH at 10°C. A solution of iodoacetic acid (530 mg) in 3 ml of acetonitrile was then added quickly to the aqueous solution with vortexing. After one minute, the organic layer was separated, dried over sodium sulfate, and evaporated to give a white solid. The product was dissolved in a few drops of chloroform and reprecipitated by the addition of 5 ml of ethyl acetate.

2-((8-[N-(2-(Iodoacetylamino)ethyl)carboxamido] octyl)thio)hexadecanoic acid (12)



This new fatty acid derivative was synthesized

bromooctanoic acid (10.65 g) with thiourea (3.65 g) in ethanol (25 ml) for 7 hours. Ten ml of a 0.1 N NaOH solution was then added and the reaction mixture was further refluxed for 3.5 hours and then allowed to cool to room temperature and stand overnight. After addition of water, the solution was extracted with ethyl acetate then acidified with 10% sulfuric acid. The organic layer was then washed with brine, evaporated to give an oily residue which was then redissolved in hexanes. A white solid appeared which was removed by filtration. The filtrate was evaporated to dryness to give the crude product. Chromatography over silica gel using 5% ethyl acetate in hexanes as the eluant afforded the pure compound.

Methyl 8-thiooctanoate (7) was prepared from the acid, 6, by esterification with excess ethereal diazomethane.

t-Butyl 2-bromohexadecanoate (8) was synthesized from 2-bromohexadecanoic acid (11.4 g) in dry t-butanol (250 ml) in the presence of dicyclohexylcarbodiimide (7.5 g, added as a solution in 50 ml of tetrahydrofuran) and 4-pyrrolidinopyridine (200 mg). The reaction mixture was allowed to stir for 2 hours at room temperature and then evaporated to dryness. The residue was heated in ether (400 ml) and solid oxalic acid (5.0 g) was introduced. The mixture was filtered and the filtrate concentrated to dryness under vacuum. The desired product was purified by recrystallization from methanol.

t-butyl 2-((8-Carbomethoxyoctyl)thio)hexadecanoate (9) was prepared by the reaction of 7 (2.82

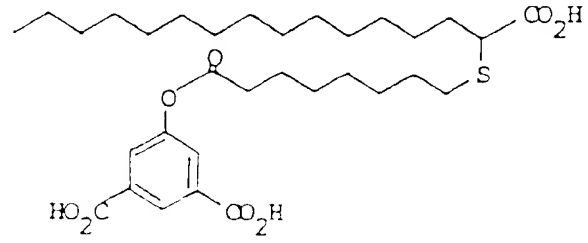
refluxed for 5 hours and then left overnight at room temperature. After concentration of the reaction mixture, addition of ice resulted in the precipitation of 9 as a white solid which was then purified by recrystallization from methanol.

Next, a mixture of 9 (0.91 g) and ethylene diamine (8.0 g) was heated at 85°C for 10 hours. The solution was then further stirred at room temperature for 72 hours. Excess ethylene diamine was removed by heating at 60°C under vacuum. The residue was diluted with water and extracted exhaustively with ethyl acetate. The organic layer was washed with water, dried with sodium sulfate and evaporated to give t-butyl 2-((8-[N-(2-aminoethyl)carboxamido]-octyl)thio)hexadecanoate (10) as a yellow oil which was carried forward without further purification.

A mixture of 10 (1.64 g) and iodoacetic anhydride (1.63 g) in 10 ml of tetrahydrofuran was stirred at room temperature for 10 hours. The solvent was evaporated under reduced pressure and the residue was redissolved in ethyl acetate. The organic layer was washed with 2% sodium bisulfite then with brine and then evaporated to dryness. The crude product was recrystallized from aqueous methanol to give t-butyl 2-((8-[N-(2-(iodoacetyl-amino)ethyl)carboxamido]octyl)thio)hexadecanoate (11). M.p. 127-128°C.

The title compound 12 was prepared by treating 11 with trifluoroacetic acid for 20 hours at room temperature to remove the t-butyl protecting group. After removing the trifluoroacetic acid under reduced pressure, the product was recrystallized

2-([8-(Carbo(3,5-dicarboxy)phenoxy)octyl]thio) hexadecanoic acid (15)

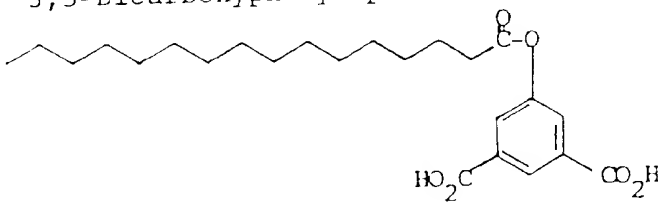


To a solution of sodium (300 mg) in 25 ml of absolute ethanol was added 6 (1.24 g) followed by addition of 8 (2.5 g). The reaction mixture was refluxed for 8 hours and left overnight at room temperature. The concentrated reaction mixture was diluted with ice water and acidified with chilled 1% acetic acid. Extraction with ethyl acetate followed by washing of the organic layer with water, drying over sodium sulfate and evaporation under reduced pressure afforded t-butyl 2-((8-carboxyoctyl)thio)-hexadecanoate (13) as a colorless oil.

To a solution of 13 (0.97 g), 4-pyrrolidino-pyridine (10 mg) and dibenzyl 5-hydroxyisophthalate (0.72 g) in 6 ml of tetrahydrofuran was added dicyclohexylcarbodiimide (0.46 g). The reaction mixture was stirred for 3.5 days after which 230 mg of dicyclohexylcarbodiimide was added and the reaction allowed to proceed for an additional day. The solvent was removed and 50 ml of ether was added followed by 260 mg of oxalic acid. The suspension was stirred for 15 minutes and then filtered. The filtrate was concentrated to give the desired product, t-butyl 2-([8-(carbo(3,5-dibenzylcarboxy)-

for 6 hours over 20% $Pd(OH)_2/C$ in cyclohexene:ethanol (1:1). The resulting compound was then treated with trifluoroacetic acid at room temperature to remove the t-butyl group.

3,5-Dicarboxyphenyl palmitate (16)



The title compound was prepared by the reaction of palmitoyl chloride (1.7 ml) with 5-hydroxyisophthalic acid (1 g) in dry tetrahydrofuran. Both starting materials were purchased from Aldrich. The reaction was allowed to proceed for 5 hours at room temperature after which the solvent was evaporated. The residue was acidified with an aqueous HCL solution containing 2.1 equivalents of acid at 0°C. The product precipatated as a white solid and was collected by filtration. After washing with cold water, it was dried under vacuum and recrystallized from benzene. M.p. 115-118°C.

For all of the compounds listed above either fast atom bombardment mass spectrometry or elemental analysis data was obtained confirming the structure.

Example 2

Reaction of Fatty Acid Protein Modifying Agents with Hemoglobin

The reactions of compounds 5, 12, 15 and 16 with native human hemoglobin (HbA) and a derivative cross-linked between the alpha subunits (HbXL99) were

9937; Snyder, S.R. et al. (1987) Proceedings of the National Academy of Sciences USA 84, 7280-7284). The reactions were carried out in 100 mM bistris buffer at pH 7.2. The compounds were first dissolved in dimethylsulfoxide at a concentration of 100 mM and diluted 100-fold into the reaction mixture to give a final concentration of 1 mM. The hemoglobin concentration during the reaction was 0.5 mM. The products of the reaction were identified and quantitated by analytical isoelectric focusing (Chatterjee, et al., 1986). In all cases the isoelectric point of the modified hemoglobin was decreased. For 5, 12 and 15 this is due to the attached negatively charged group. With 16 (and also 15) the decrease in isoelectric point is due to the loss of the positive charge of the modified amino group.

Reactions of 15 and 16 with oxyHbA and oxyHbXL99 were carried out at 37°C for 4 hours. Very little modification of the protein was observed (less than 5%). Substantially higher temperatures or longer reaction times could not be studied because of the susceptibility of oxyhemoglobin to autooxidation. The reaction of 15 and 16 with deoxyHbA and deoxyHbXL99 afforded better results. After incubation for 4 hours at 55°C each compound gave about 20% yield of a specific reaction product with both native and the cross-linked hemoglobin. The reaction with both hemoglobins was blocked by inositol hexaphosphate (IHP). IHP binds very tightly to deoxhemoglobin at the 2,3-diphosphoglycerate binding site, indicating that these compounds react with an amino group of the protein in

are sufficient to direct these reagents to react with deoxyhemoglobin at the beta cleft. The yield of the reaction can be further improved by incorporating additional negatively charged substituents into the aromatic ring.

Compounds 5 and 12, both alkylating agents, reacted selectively with deoxyHbA to yield one major product. The level of modification obtained after reaction for 6 hours at 37°C was 75% and 40%, respectively. Similar results were obtained for the reaction of these compounds with deoxyHbXL99.

The reactions of deoxyHbA with 5 and deoxyHbXL99 with 16 were performed on a larger scale to isolate the fatty acid modified derivatives for plasma half-life studies. The reactions were carried out under the same conditions as described above with 2 to 4 g of hemoglobin. The derivative modified with 16 was purified by chromatography over DEAE-Sepharose (Pharmacia). Using 0.2 M glycine pH 8.0 as the column buffer (see Chatterjee et al., 1986), the desired product was eluted with 0.1 M NaCl. The product of the reaction of deoxyHbA with 5 was isolated by preparative isoelectric focusing in a pH 6.7 to 7.7 gradient. Ampholines were obtained from Pharmacia.

Example 3

Prolongation of the Intravascular Retention Time of Hemoglobins Modified with a Long Chain Fatty Acid

Transfusion experiments were carried out in the rat to measure the plasma half-life of the two fatty acid modified hemoglobin derivatives just described.

filtration into lactated Ringer's and concentrated to 7% (approximately 1 mM). Male Sprague-Dawley rats weighing about 300 g were anesthetized with urethane at a dosage of 1.2 g per kg body weight. A catheter was placed in the femoral artery and 15% of the total blood volume was removed (about 3cc) and replaced with an equal volume of the hemoglobin solution. Blood samples were withdrawn at subsequent times and the plasma hemoglobin concentration was determined spectrophotometrically.

In a first series of experiments the intravascular retention times of unmodified hemoglobin and the fatty acid derivative of HbA prepared with 5 were compared. The plasma half-life observed for HbA was about 1.3 hours similar to that reported previously for this model (Snyder, S.R. et al., 1987). The half-life of the fatty acid modified derivative was prolonged to 2.8 hours. The plasma half-life of hemoglobin is highly dose and species dependent. A half-life of about 3 hours under these conditions in the rat would translate to approximately 20 hours in man at a 50% blood volume replacement.

In a second series of experiments the intravascular retention times of HbXL99 and HbXL99 further modified with the fatty acid derivative 16 were compared. The plasma half-life of HbXL99 was found to be 3.2 hours as reported earlier (Snyder, S.R., et al., 1987). Renal excretion of HbXL99 is almost completely blocked due to the intramolecular cross-link between the alpha chains. The plasma half-life of the fatty acid modified derivative was increased about 2-fold to 6 hours

intermolecularly (Snyder, S.R., et al., 1987) consistent with the fact that such a dimer and the complex formed between HbXL99 and albumin have essentially the same size and molecular weight, about 130,000 daltons.

These results demonstrate that the binding of protein drugs modified with fatty acid derivatives to albumin not only inhibits their excretion by the kidneys but also slows their egress across other capillary beds. It is also apparent from these studies that in preparing fatty acid modified derivatives of hemoglobin for use as blood substitutes it is desirable to use a hemoglobin that is also cross-linked intramolecularly.

What is claimed is:

1.

A peptide and protein drug carrier composition which decreases metabolism and/or excretion of the drug, said composition comprising:

a peptide/protein drug chemically linked to an apolar bridging modifier which in turn is bound noncovalently to albumin.

2.

The composition of claim 1 wherein the bridging modifier is a long chain fatty acid or a derivative thereof.

3.

The composition of claim 2 wherein the long chain fatty acid hydrocarbon chain is from about C_{10} to about C_{24} in length.

4.

The composition of claim 3 wherein the hydrocarbon chain is from C_{16} to C_{20} in length.

5.

The composition of claim 1 wherein the bridging modifier has a functional group incorporated therein to provide an attachment means to the drug.

6.

The composition of claim 5 wherein the functional group is selected from the group consisting of activated carboxylic acid derivatives including anhydrides, N-hydroxysuccinimide esters, and phenylesters; alkylating agents such as alkyl halides, alpha-halo carboxylic acids and amides, and N-substituted maleimides; and carbonyl containing agents such as aldehydes and ketones.

7.

8.

The composition of claim 7 wherein the bridging modifier is attached to hemoglobin only at the 2,3-diphosphoglycerate binding pocket.

9.

The composition of claim 7 wherein the bridging modifier is attached only at the SH group of cysteine residues of the drug.

10.

The composition of claim 1 wherein the protein drug is selected from the group consisting of superoxide dismutase, soluble CD4 protein, insulin, gamma-interferon, erythropoeitin, growth hormone, tissue plasminogen activator, hemglobin and derivatives of hemoglobin cross-linked intramolecularly.

11.

A method of modifying protein and peptide drugs so that drug retention time in the systemic system is increased, said method comprising:

(a) reacting a protein or peptide drug with an apolar bridging modifier; and thereafter,

(b) reacting the product of step (a) with albumin to provide a composite of the protein or peptide drug linked to albumin as a carrier, said link being through said bridging modifier.

12.

The method of claim 11 wherein the bridging modifier is a long chain fatty acid or a derivative thereof.

13.

The method of claim 12 wherein the fatty acid derived modifier has a C_{12} to C_{20} chain length.

14.

The method of claim 11 wherein the bridging modifier is site specifically attached to said protein or peptide drug, as opposed to random site attachment.

15.

The method of claim 14 wherein the protein drug is selected from the group consisting of those having a molecular weight below about 60,000.

16.

A method of treating patients with a protein or peptide drug to increase systemic retention time, said method comprising:

delivery to the systemic system of said patient a protein or peptide drug which is bound to albumin as a carrier molecule through linkage of both said protein or peptide drug and said albumin to a fatty acid derived bridging molecule.

17.

The method of claim 16 wherein the protein drug and fatty acid derived bridging molecule are administered to the systemic system wherein binding to said albumin occurs endogenously.

18.

The method of claim 17 wherein the protein drug has a molecular weight of below about 60,000.

19.

The method of claim 17 wherein the fatty acid derived molecule has a chain length of C_{10} to C_{24}.

20.

The method of claim 19 wherein the fatty acid derived molecule has a chain length of $C_{\ldots}$ to $C_{\ldots}$

21.

The method of claim 16 wherein the duration of activity of said protein or peptide drug is controlled by administering an apolar compound which competes with said bridging molecule for binding sites on albumin and thereby terminates the action of the drug.

22.

The method of claim 21 wherein said apolar compound is a long chain fatty acid or derivative thereof.

23.

The method of claim 22 wherein the fatty acid derived molecule has a chain length of C_{16} to C_{20}.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/03889

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 A 61 K 47/48

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	Chemical Abstracts, vol. 92, 1980, (Columbus, Ohio, US), W. Scheider:" Ligand-independent activated state of serum albumin for fatty acid binding", page 194, column 1, abstract No. 175994d, & J. Phys. Chem. 1980, 84 (8), 925-8, see abstract	1-15
Y	EP,A,0359428 (R.E. OFFORD) 21 March 1990, see the whole document, especially column 3, lines 18-35	1-15
A	EP,A,0326618 (NIPPON HYPOX LABORATORIES INC.) 9 August 1989, see the claims	1-15
A	EP,A,0037388 (INSTITUT INTERNATIONAL DE PATHOLOGIE CELLULAIRE ET MOLECULAIRE) 7 October 1981, see the claims	1-15

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30-09-1991	07 11 91

MORTENSEN

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1 [X] Claim numbers 16-23 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv):
methods for treatment of the human or animal body
by surgery or therapy, as well as diagnostic methods.

2 [X] Claim numbers 1-15(incompletely) because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The term "apolar bridging modifier" used in claims 1 and 11
is not a sufficiently well defined term to enable full
understanding of the subject matter for which protection is being sought. From interpreration of the examples and claims this term has been understood to mean "a long chain fatty acid", and thus the scope of the search was restricted to drug-albumin conjugates containing such long chain fatty acid apolar bridging modifiers. See PCT Art. 6 .../...

3 [ ] Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1 [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2 [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3 [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4 [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9103889 SA 48398

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/10/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0359428	21-03-90	AU-A-	4190689	23-03-90
		WO-A-	9002136	08-03-90
		JP-T-	3501623	11-04-91
EP-A- 0326618	09-08-89	JP-A-	63215642	08-09-88
		JP-A-	63215640	08-09-88
		WO-A-	8806457	07-09-88
EP-A- 0037388	07-10-81	BE-A-	882541	16-07-80
		JP-A-	57018624	30-01-82